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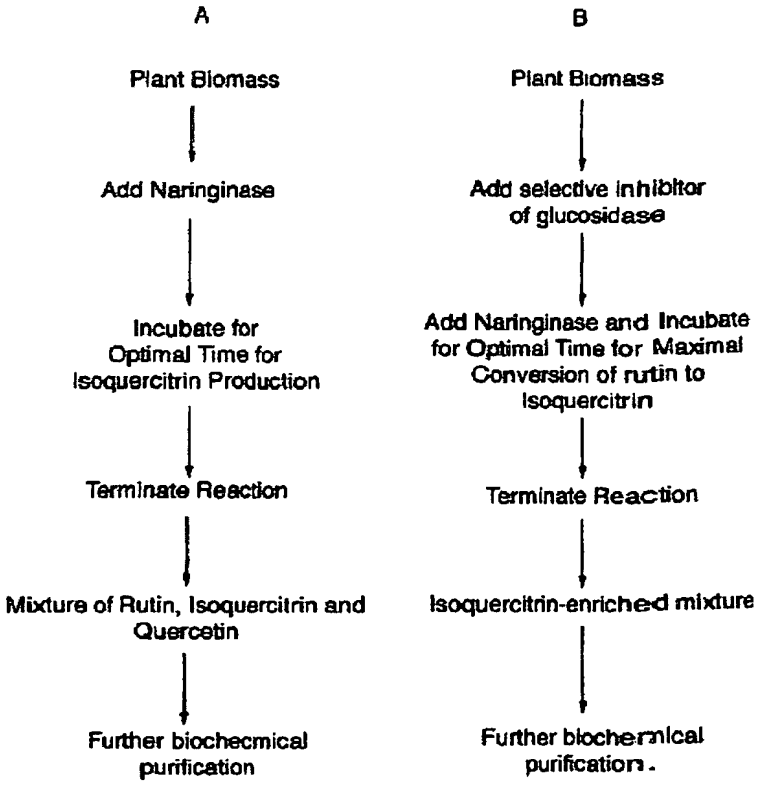

(57) Abstract: A process for preparing a rutin-enriched composition from plant biomass comprises extraction with an aqueous solution, and precipitation. An enzyme preparation, such as naringinase, is used for the transformation of rutin to higher value compositions containing increased proportions of isoquercitrin and quercetin.

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EXTRACTION, PURIFICATION AND CONVERSION OF FLAVONOIDS FROM PLANT BIOMASS

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EXTRACTION, PURIFICATION AND CONVERSION OF FLAVONOIDS FROM PLANT BIOMASS

This invention relates to flavonoids and in particular to rutin enriched compositions prepared from plant biomass, which can be enzymatically converted to the more valuable flavonoids isoquercitrin and quercetin.

Background of the Invention

Plant flavonoids usually occur in plants as glycosides, although in some circumstances they may occur as free aglycones. Most glycosides are O-glycosides, with the most common monoglycoside being at the 7-position. Diglycosides usually have sugars at the -7 and -3 positions and occasionally the -7 and -4' positions. Other combinations and mono-O-glycosides exist but are less abundant. C-glycosides also occur in a more restricted distribution with C-6 and C-8 glycosides being the most common (Harbone, 1994).

Plant flavonoids have antioxidative properties (Bors *et al.*, 1990), cytostatic effects in tumorigenesis, and the ability to inhibit a broad spectrum of enzymes, such as angiotensin converting enzyme (ACE), protein kinase C, tyrosine protein kinase, and topoisomerase II. They are regarded as potential cancer preventatives and cardioprotective agents (Manach *et al.*, 1996; Skibola and Smith, 2000). Their potential use as anti-inflammatory or antiviral agents has also been examined (Middleton and Kandaswami, 1993). Backhaus (1995a) claimed that bioflavonoids, especially rutin, citrin, quercetin, hesperidin or derivatives were responsible for the inactivation of protein-cleaving enzymes (such as hyaluronidase and/or collagenase), which promote skin-aging processes. These compounds may be used for general skin care or cosmetic surgery. It is reported that rutin, quercetin, isoquercitrin, catechin and other compounds also prevent and ameliorate the aging phenomena of the skin (Arata, 1992). Midori (1994) claimed that, together, quercetin glycoside, divalent

metal ion, and extract of liquorice prevent intoxication by promoting alcohol metabolism in the human liver.

Rutin is a flavonoid glycoside comprised of quercetin and the sugar, rutinose. Many beneficial health effects of rutin have been demonstrated. Such effects have been attributed to anti-inflammatory, anti-mutagenic, anti-tumor, anticarcinogenic, smooth muscle relaxation, and estrogen receptor binding activities of rutin (Pisha and Pezzuto, 1994). Rutin is also being used in the treatment of capillary fragility, cerebral thrombosis, retinitis and rheumatic-fever-associated haemorrhagic conditions (Griffith *et al.*, 1944; Matsubara *et al.*, 1985; Iwata *et al.*, 1990; Yildzogle-Ari *et al.*, 1991). Under conditions of low dietary fat intake, rutin and quercetin have been reported to considerably suppress colon tumor incidence (Agullo *et al.*, 1994; Deschner, 1992). Backhaus (1995b) claimed that rutin and its derivatives, in an oral dosage form, and injection or infusion solution, or a suppository, would inactivate retroviruses (e.g. HIV). Rutin can be used as a natural coloring agent, an oxidation inhibitor, vitamin, sunburn preventative in cosmetics (rutin will absorb ultra violet rays), and as an ingredient in functional food applications (Anonymous, 1990a,b).

Rutin can be found in many plants including buckwheat (leaves, flowers, stems, straws, hulls, and groats), Japanese pagoda tree *(Sophora japonica)*, tomatoes, pansies (*Viola* sp., *Violaceae*), tobacco, forsythia, hydrangea, fava d'anta (*Dimorphandra gardnerina* and *Dimorphandra mollis*) and eucalyptus (Humphreys, 1964). Buckwheat is considered to be capable of providing a major dietary source of rutin. Kitabayashi *et al.* (1995) reported that the rutin content of buckwheat seed ranges from 0.126 to 0.359 mg/g dry weight. Oomah and Mazza (1996) reported 0.47 and 0.77 mg/g dry weight of rutin in whole seed and hulls, respectively. They also reported that flavonoids were highly concentrated in the hulls; the mean flavonoid content of buckwheat seeds and hulls were 3.87 and 13.14 mg/g, respectively. Prochazka (1985) reported that 6% rutin (wt/wt) was found in carefully dried, Czechish buckwheat leaves at the flowering stage. Dry herbage yields were 600 to 1000 kg/ha, which at 4% (wt/wt) rutin concentration, amounted to 24-40 kg rutin/ha.

Although most of the details of the industrial production of rutin are proprietary and not described in the open literature, we are aware that Merck GmbH extracts rutin from fava d'anta for commercial purposes. Heywang and Basedow (1992) of Merck GmbH Germany, extracted rutin from shoots of fava d'anta (*Dimorphandra*) with 1,4-dioxane under reflux. Rutin was recovered by crystallization at room temperature. Dioxane is, however, considered carcinogenic.

Huo (Chinese Patent 1217329, 1999) described an extraction of rutin from tartary buckwheat seeds by washing with water, coarse grinding, coarse screening, soaking in water, drying in the air, fine grinding, soaking in edible alcohol, extracting below 60°C, and filtering. Balandina *et al.* (1982) extracted rutin from buckwheat seeds with hot water to remove the desired product and crystallized it.

Zhai (Chinese Patent CN 1160048, 1997) described the extraction of rutin from *Flos sophorae* by soaking with saturated limewater containing 1 - 10% borax, and precipitating at pH 1- 6 by adding HCl.

Matsumoto and Hamamoto (1990) recovered rutin from *Sophora augustifolia* buds with methanolic extraction, adsorption onto activated carbon followed by desorption, by elution with 1% ammonia in 40% ethanol, and recrystallization from 20% ethanol.

Liu (1991) described a method of extracting rutin from Japanese Pagoda tree (*Sophora japonica*) buds by pulverizing, streaming in limewater, neutralizing the supernatant, cooling, filtering, washing, and drying the precipitates. The yield was 14.2% (wt/wt) and the product contained 95.1% (wt/wt) rutin.

Sloley *et al.* (2000) reported that, while hypericin is regarded as a marker chemical for extracts of leaves and flowers of *Hypericum perforatum* (St. John's wort), other compounds such as hyperforin, hyperoside, rutin and quercetin are presented in much higher concentrations. They also found that chemical composition profiles varied greatly among different extracts. However, free-radical-scavenging capacity

correlated positively to quercetin content. The averaged rutin and quercetin contents in sixteen St. John's wort extracts were 2.0 and 0.3% (wt/wt), respectively.

One gram of rutin can be dissolved in about 8 L of water at room temperature or 200 ml of boiling water. Zirlin (U.S. Patent 3,822,475, 1974) disclosed a method for prevention of crystallization of sparingly soluble flavonoids in acidic soft drinks. Flavonoids are mixed with sucrose and heated to a caramel melt stage (140 - 185°C), dissolved in an aqueous system, and the water is evaporated off to obtain the dry mixture.

Quercetin glycosides were modified to water-soluble flavonol glycosides with alpha-glucosidase (E.C. 3.2.1.20), cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19), alpha-amylase (E.C. 3.2.1.1), glucoamylase (E.C. 3.2.1.3), beta-amylase (E.C. 3.2.1.2), and galactose-transferring enzymes (β-galactosidases) as described by San-Ei Chemical Industries Ltd. and Hayashihara Biochemical Laboratory Inc. (Nishimura *et al.*, 1992; Suzuki *et al.*, 1992a,b; Suzuki *et al.*, 1995; Suzuki *et al.*, 1996; Washino 1992; Yoneyama *et al.*, 1996). Hayashihara Biochemical Laboratory Inc. claimed that they succeeded in producing water-soluble rutin which water solubility was increased by more than five thousand times (Anonymous, 1990a).

Before 1990, quercetin was considered to be mutagenic and carcinogenic (Manach *et al.*, 1996). Metabolic animal studies have shown that quercetin may be rapidly converted to the non-mutagenic 3'-O-methylquercetin metabolites (Morand *et al.*, 1998; Skibola and Smith 2000). More importantly, quercetin is reported to have antibacterial, antiviral, antioxidant, antiproliferative, ant-inflammatory, and anticarcinogenic effects (Crespy *et al.*, 1999; Skibola and Smith, 2000).

Quercetin has also shown powerful inhibitory activity on various tumor cells (Middleton and Kandaswami, 1993; Caltagirone *et al.*, 2000), colon cancer cells (Agullo *et al.*, 1994; Deschner, 1992) and ulcers (Borrelli and Izzo, 2000). Quercetin has been identified as a potent topoisomerase II inhibitor at low concentrations,

similar in activity to the epipodophylotoxins widely used in cancer therapy (Skibula and Smith, 2000).

Ishige *et al.* (2001) showed that many flavonoids and related polyphenolic compounds protected the mouse hippocampal cell line HT-22 and rat primary neurons from oxidative stress caused by glutamate. This finding is significant because nerve cell death from oxidative stress has been implicated in a variety of pathologies, including stroke, arteriosclerosis, trauma, and Alzheimer's and Parkinson's diseases. Their data show that some flavonoids (quercetin, kaempferol, and fisetin) are quite protective, while others (rutin, chrysin, and apigenin) are inactive. Quercetin alters glutathione (GSH) metabolism and inhibits reactive oxygen species (ROS) in a cell culture model of oxidative stress. Its mechanism of action is similar to that of propyl gallate and methyl caffeate, but different from that of vitamin E. Noroozi *et al.* (1998) reported that quercetin is more potent than rutin and vitamin C in countering against oxidative DNA damage.

Ashida *et al.* (2000) reported that dietary flavonols (quercetin and rutin) and flavones suppress antagonistically the transformation of aryl hydrocarbon receptor (AhR) induced by dioxin. Quercetin is more potent that rutin in counteracting the toxicity of this environmental contaminant. In the area of anticarcinogenicity, phase I enzymes oxidize, reduce or hydrolyze carcinogens, and phase II enzymes conjugate or otherwise affect carcinogens. Valerio *et al.* (2001) demonstrated that quercetin is a phase II enzyme inducer that stimulates phase II detoxifying activities. Phase II enzymes can also scavenge strong oxidants, and scientific interest has been directed toward their activity as a means of decreasing the risk of cancer. Use of phase II enzyme inducers, many of which are found in common foods, is one way to increase phase II enzyme activities in body tissues.

Agullo *et al.* (1997) reported that quercetin was an effective inhibitor of phosphatidyl inositol 3-kinase (PI 3-kinase; an enzyme involved in cell multiplication and transformation). Luteolin, apigenin and myricetin also exhibit such activity. Inhibition of PI 3-kinase may be linked to the antitumor properties of these

flavonoids. Also, quercetin was reported to inhibit lymphocyte tyrosine kinase activity, and exhibit antitumor properties in a phase I clinical trial (Ferry *et al.* 1996).

Watanabe *et al.* (1997) reported that quercetin is responsible for the alpha-glucosidase inhibitor activity of tochu (*Eucommia ulmoides*) leaves. Since alpha-glucosidase is an enzyme that catalyzes a final step in the digestive process of carbohydrates, the implication of the above finding is that quercetin may suppress postprandial hyperglycemia and could be used for the treatment of diabetes with potential application of late diabetic complication, obesity and related disorders. Quercetin also blocks an enzyme that leads to accumulation of sorbitol, which has been linked to nerve, eye and kidney damage in those with diabetes. However, no human research has evaluated the possible beneficial effect of quercetin for diabetics (Wang, 2000).

Kato *et al.* (1983) showed that in mice or rats receiving 0.5% quercetin in their diets there was a significant lowering of serum triglycerides. Supplementation of quercetin was also shown effective in blunting the rise of serum and liver cholesterol in rats fed a high cholesterol diet (Basarkar, 1981)

Quercetin and its glycoside extracted and purified from the leaf of *Alpinia urarensis* Hay showed blood platelet aggregation-inhibition activity. Its activity was greater than that of aspirin or ginseng saponins as control blood platelet aggregation inhibitors (Okuyama et al., 1996).

In Japanese patent publication No. 06248267, Nakayama (1994) claimed that quercetin, kaempferol, catechin or taxifolin can be used in food or as pharmaceuticals for prevention and treatment of diseases caused by malfunction or scavenging action, ischaemic disease, rheumatism, diabetes etc.

Lutterodt and Abu Raihan (1993) reported that quercetin has narcotic-like antinociceptive activity that interferes with pain transmission. A dose of 50 mg of quercetin/Kg body weight would have the same effect as that of 2.5 mg of morphine sulfate/Kg.

Naturally occurring isoquercitrin (quercetin-3-O-beta glycoside) can be extracted from flowers of levant cotton (*Gossypium herbaceum*), *Waldsteinia fragarioides* (Michx) Tratt (*Rosaceae*), *Spartium junceum* L. (*Fabaceae*) (Yesilada *et al.*, 2000), and horse chestnut (*Aesculus hippocastanum*). It is also found in celery seed, fennel seeds, horsetail, red clover and St. John's wort. Isoquercitrin has shown to possess several biological activities, including inhibition of angiotensin converting enzyme (ACE), inhibition of prostaglandin synthesis, and antiviral activity (Abou-Karam and Shier, 1992).

The role of bacterial enzymes in the digestive absorption of flavonoids is important because mammalian tissues are unable to synthesize such hydrolases. Griffiths and Barrow (1972) have shown that flavonoid glycosides ingested by germ-free rats were recovered unhydrolyzed in the feces. Hydrolysis of the sugar-aglycone bond takes place in the distal ileum and the caecum.

During absorption across the intestinal membrane, flavonoids are absorbed in the aglycone and/or glucoside forms and are partly transformed into their glucuronides, sulfates or methoxylates (Manach *et al.*, 1998). Free quercetin could not detected in blood plasma. The small fraction of flavonoids that is absorbed is metabolized by liver enzymes resulting in polar conjugates being excreted in the urine or returned to the duodenum via the gallbladder. The largest fraction of ingested flavonoids, that is not absorbed, is degraded by the intestinal microflora. The bacterial enzymes catalyze several reactions, including hydrolysis, cleavage of the heterocyclic oxygen-containing ring, dehydroxylation, and decarboxylation. Several phenolic acids are produced, depending on the structure of the flavonoid involved. Phenolic acids can then be absorbed and subjected to conjugation and O-methylation in the liver and may then enter into the circulation (Manach *et al.*, 1996).

Crespy *et al.* (1999) demonstrated that quercetin and isoquercitrin are much more bioavailable than rutin. Rutin is absorbed more slowly than quercetin, isoquercitrin and isorhamnetin because it must be hydrolyzed by the caecal microflora, whereas

quercetin, isoquercitrin and isorhamnetin are absorbed from the small intestine (Manach *et al.*, 1997). Morand *et al.* (2000) also showed that isoquercitrin is better absorbed than other quercetin forms (quercetin, rutin and quercitrin). Four hours after a meal, the metabolites identified in hydrolyzed plasma were 3'- and 4'-methylquercetin regardless of what form of quercetin was consumed. However, the total concentration of metabolites in the plasma was markedly different: 33.2, 11.2, and 2.5 μM for the isoquercitrin, quercetin and rutin, respectively. After consumption of quercitrin (quercetin 3-rhamnoside), they failed to detect any metabolites in the plasma. Gee *et al.*, 2000 showed that isoquercitrin passes across the small intestinal epithelium more rapidly than free quercetin aglycone. These data established a ranking of flavonoid bioavailability as isoquercitrin > quercetin > rutin.

Naringinase is an enzyme preparation that can be produced from cultures of *Penicillium aspergillus*, *Coniella diplodiella*, *Cochliobolus miyabeanus*, *Rhizoctonia solanii*, *Phomopsis citri*, and *Penicillium decumbens*. Most commercial naringinase preparations were produced from *Penicillium decumbens*. Narikawa *et al.* (1998) concluded that *Penicillium decumbens* does degrade rutin, but their work was qualitative in nature, and they did not indicate what the results of that degradation were.

Naringinase is used to hydrolyze narigin, 7-(2-rhamnoside-beta-glucoside) of 4', 5, 7 – trihydroxyflavonone, to narigenin. It is used commercially to reduce the bitter taste in citrus fruit or juice. Naringinase was used by Uyeta *et al.* (1981) during an investigation of tea infusions. The effect of naringinase treatment on the mutagenic activity of tea infusions was similar to that of treatments with acid or hesperidinase. However, they neither characterized nor identified the hydrolyzed products. They did identify kaempferol, quercetin and myricetin as mutagenic principles in tea infusions treated with human faecal bacteria.

Although isoquercitrin would appear to be the most desirable quercetin derivative, there are currently no concentrated or pure forms of this compound available in the market place – other than very small amounts for use as analytical standards. There is

no previously disclosed method for the processing of buckwheat foliar material for recovery of flavonoids and for the further biotransformation of such flavonoids to highly bioavailable, performance-enhanced, high-value products such as isoquercitrin and quercetin. Previously disclosed are only classical laboratory procedure for the extraction and purification of rutin.

Usually, the concentrations of naturally occurring isoquercitrin and quercetin found in biological systems are much lower than that of rutin. Isoquercitrin and quercetin extracted from biological systems demand much higher prices due to their rareness and bioavailability. There is not presently any commercially feasible technology for the biotransformation of rutin (regardless of the source) to highly bioavailable, performance enhanced and high value products such as isoquercitrin and quercetin.

Summary of the Invention

It is an object of this invention to provide an isoquercitrin-enriched composition derived from rutin, and to provide such a composition economically in commercial amounts sufficient to permit their use in functional foods, nutraceutical, natural health products, cosmetics and pharmaceutical applications.

It is a further object of this invention to provide a composition derived from rutin that is enriched in controlled proportions of isoquercitrin and quercetin, and to provide such a composition in commercial amounts sufficient to permit their use in functional foods, nutraceutical, natural health products, cosmetics and pharmaceutical applications.

It is a further object of this invention to provide a method whereby the yield of isoquercitrin can be maximized by inhibiting the conversion of isoquercitrin to quercetin. In the invention this is accomplished by the addition of an inhibitor of the ß-D-glucosidase activity present in naringinase preparations.

It is a further object of this invention to provide a process for deriving rutin from buckwheat, and in particular to provide such a process deriving rutin from the

buckwheat plant residue that remains in the field after buckwheat seed has been harvested, thereby converting a cheap waste product into a more valuable product.

In a first aspect the present invention provides a process for preparing a rutin enriched composition from biomass containing rutin, the process comprising performing a flavonoid extraction process on the biomass using an aqueous solution; filtering the solution to produce an extract solution; allowing the extract solution to stand such that a precipitate forms; collecting and drying the precipitate to form the rutin enriched composition.

Preferably the aqueous solution is maintained at a temperature above 30°C during the extraction process. Preferably the aqueous solution is an aqueous alcohol solution, with an alcohol concentration of greater than 20% alcohol by volume, and for best results between 50% and 100% alcohol by volume. The extract solution is preferably concentrated to about one fifth to one tenth its original volume, and then chilled while standing to facilitate precipitation.

With the process of the present invention, a rutin enriched composition having 70% rutin content by weight can be prepared through relatively simple wet chemistry means and without the necessity of chromatographic means. Most economically the crop residue left after seeds have been harvested from a field of buckwheat is used to provide the rutin containing biomass. This residue has formerly had little if any value. Use of this crop residue is preferred over prior art use of buckwheat at the flowering stage since the seeds can be harvested, providing the primary return from a buckwheat crop. In the prior art the total return from a buckwheat crop is derived by purchasing it at the flowering, or other premature stage, as a feedstock for rutin production.

In a second aspect the invention provides a composition enriched in isoquercitrin prepared by a process comprising providing a solution having rutin suspended therein at conditions suitable for enzyme incubation; adding an enzyme preparation comprising naringinase to the solution; maintaining the conditions of the solution

suitable for enzyme incubation during an incubation period; terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme activity. These changes include lowering the pH and increasing the temperature of the solutione. Adjusting the duration of the incubation period controls the proportion of isoquercitrin in the composition.

In a third aspect the invention provides a composition enriched in isoquercitrin prepared by a process comprising providing a solution having rutin suspended therein at conditions suitable for enzyme incubation; adding an enzyme preparation comprising the enzymes naringinase or α-L-rhamnosidase to the solution; maintaining the conditions of the solution suitable for enzyme incubation during an incubation period; terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme incubation. For optimal yields, the temperature should be in the range of 50 – 55°C and should not exceed 65°C. Adjusting the duration of the incubation period controls the proportion of isoquercitrin in the composition. The incubation period is optimally in the range of 1 – 48 hrs. Lowering pH and increasing the temperature of the solution terminates the incubation period by denaturing the enzyme preparation.

The proportion of isoquercitrin in the composition can be up to about 95%. The enzyme incubation with the enzyme preparation containing α-L-rhamnosidase and β-D-glucosidase also converts rutin to quercetin. The incubation period can be adjusted to provide a composition enriched with both isoquercitrin and quercetin in varying proportions.

Conveniently and economically the enzyme preparation can be naringinase, which is commercially available and economical. Naringinase is sold with a guaranteed content of the enzyme β-D-glucosidase for various commercial uses. Contrary to the prior art revealed by Narikawa *et al* (1998), it was found that naringinase from *Penicillium decumbens* was able to cleave sugar from the rutin.

Incubation of the enzyme α-L-rhamnosidase with rutin converts the rutin to isoquercitrin. Incubation of the enzyme β-D-glucosidase with isoquercitrin converts isoquercitrin to quercetin. Naringinase contains both the enzyme α-L-rhamnosidase and the enzyme β-D-glucosidase, and is commercially available in economic quantities.

An efficient, economic and commercially viable biotransformation can be accomplished without using purified or other expensive forms of α-L-rhamnosidase and β-D-glucosidase. Compositions with different ratios of rutin/isoquercitrin/quercetin can be tailor-made by manipulating biotransformation conditions. The process of the present invention produces a product of highly concentrated rutin, isoquercitrin, quercetin, or mixtures thereof, which may then subsequently be purified using standard biochemical purification techniques.

In a fourth aspect of the invention, a β-D-glucosidase inhibitor is added to the rutin solution prior to the addition of the naringinase enzyme. In the preferred embodiment, the β-D-glucosidase inhibitor is D-Δ-gluconolactone. By inhibiting the β-D-glucosidase component of the naringinase preparation, isoquercitrin is not converted to quercetin; with the result that isoquercitrin is obtained at high yield, and at purity greater than 80%.

The process of the present invention can be use to produce a product of highly concentrated rutin, isoquercitrin, quercetin or mixtures thereof from a variety of plant biomass sources, including, but not limited to members of the genus *Fargopyrum*, leaves of St. John's Wort; ginkgo; biloba; alfalfa; mulberry; algae; apple peel; pear peel; onion skin; asparagus tip; and rose pericarps.

The isoquercitrin-enriched product produce by the process of the present invention has bioactive properties including angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory,

tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

The bioactive properties of the isoquercitrin-enriched product produce by the process of the present invention will be useful as an additive in health foods, pharmaceuticals products, nutraceuticals and cosmetics. When added to products, the bioactive properties will be useful in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

The present invention also offers processing and product flexibility enabling economical manufacture and satisfaction of market preferences.

These and other objects, features, and advantages of the invention become further apparent in the following detailed description of the invention that illustrates, by way of example, the principles of this invention.

Brief Description of the Drawings

While the invention is claimed in the concluding portions hereof, preferred embodiments are provided in the accompanying detailed description which may be best understood in conjunction with the accompanying diagrams where like parts in each of the several diagrams are labeled with like numbers, and where:

Fig. 1A illustrates the chemical structural formula for rutin;

Fig. 1B illustrates the chemical structural formula for isoquercitrin;

Fig. 1C illustrates the chemical structural formula for quercetin;

Figs. 2A – 2C show the results of HPLC analysis of: (2A) Methanol extract of buckwheat leaves (RT: 14.862 = rutin, RT: 20.947 = quercetin); (2B) Precipitate obtained from the aqueous alcohol extract of buckwheat leaves that had been concentrated to the aqueous phase and chilled (RT: 14.785 = rutin). (2C) Conversion of rutin to isoquercitrin (RT: 15.181) and quercetin (RT : 20.372) after 24 hour incubation with naringinase. All samples were chromatographed on a C-18 Symmetry column eluted with a water:acetonitrile gradient containing 0.05% Trifluoroacetic acid. The column efluent was monitored at 280 nm and dissolved solids were quantified by ELSD.

Figs. 3A – 3C show the results of HPLC analysis of rutin samples: (3A) Commercial rutin sample (Street Chemicals) (RT: 14.875 = rutin, RT: 15.442 = isoquercitrin); (3B) Precipitate recovered after naringinase treatment of rutin (RT: 15.487 = isoquercitrin, RT: 20.843 = quercetin); (3C) Purified isoquercitrin obtained by preparative HPLC (RT: 15.436 = isoquercitrin). All samples were chromatographed on a C-18 Symmetry column eluted with a water:acetonitrile gradient containing 0.05% Trifluoroacetic acid. The column effluent was monitored at 280 nm and dissolved solids were quantified by ELSD.

Fig. 4 is a summary flowchart of two methods of practicing the present invention. Using method A, rutin is recovered from plant biomass and then converted to a mixture of rutin, isoquercitrin and quercetin. Using method B, the addition of an inhibitor of β-D-glucosidase is added to prevent the conversion of isoquercitrin to quercetin. Using method B, the yield and purity of isoquercitrin by the method of the present invention is enhanced.

Detailed Description of the Illustrated Embodiments

The present invention provides a method for the production of high value bioavailable flavonoids from plant biomass. As described above, flavonoids have been shown to have a range of useful bioactive properties. One of the problems in the use of flavonoids in therapeutic applications is that they normally exist at low concentrations in nature. In order to use flavonoids as additives in pharmaceutical, nutraceutical or other health products, a method for purifying flavonoids is required.

In the present invention, the flavonoid rutin is recovered by standard biochemical methods. Rutin is then converted to isoquercitrin and quercetin through the action of the enzyme preparation naringinase. A further refinement of the present invention shows that the yield of the intermediate product isoquercitrin can be enhanced by selectively inhibiting the ß-D-glucosidase activity present in the naringinase preparation, using the food additive d-Δ-gluconolactone.

The following examples and figures illustrate the operation of certain embodiments of the present invention so that it may be more readily understood.

With specific reference now to the figures in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice. It is stressed that the particulars shown are by way of example and for the purposes of illustrative discussion,

Extraction of Rutin from Plant Biomass

Examples 1 and 2 below demonstrate that rutin in plant biomass can be recovered by a process involving extraction in aqueous solution, concentration and precipitation. It is contemplated that the step of concentrating the extract solution could be omitted, however that relatively simple and economical step increases the efficiency of the process.

As shown in Example 1, extraction in hot water as described recovered 36% of the available rutin from the leaves.

As shown in Example 2, extraction in an aqueous alcohol solution with 50% methanol by volume as described recovered 65% of the available rutin from the leaves.

As shown in Example 3, the rutin content of the rutin enriched composition can be increased to about 70% by simple wet chemistry means without using chromatography.

As shown in Example 4, the extraction efficiency of the process of the invention varies with the alcohol concentration, the temperature of the aqueous solution, the solid to solvent ratio, and the extraction time. For economic commercial processes, a suitable combination of these variables can be determined based on the economics of providing them.

It is anticipated that the extraction could be conducted in either a sequential batch or continuous feeding mode. The extraction recovery ratio of the process might also be improved by adding the extracted biomass to a fresh quantity of solvent and running second or additional extractions.

Prior art in this area was mostly focused on analytical methodology of flavonoids, concentration and quantity of flavonoids from biomass. Rutin enriched fractions from

precipitation have not earlier been described as a finished product. The value of a rutin-enriched composition has not earlier been recognized.

Example 1:

Aqueous Extraction, Concentration and Precipitation of Rutin from Buckwheat Leaf Material

Following harvest and drying, buckwheat leaves were prepared for extraction by grinding on a Wiley mill to pass a 2mm screen. One kg of ground buckwheat leaves (rutin content is 3.74%, dry weight basis) were extracted in 10 L of water with continuous stirring at 90°C for 1 hour. The resulting suspension was filtered, and the filtercake was washed 2 times with 300 ml of hot (95°C) water. The wash filtrate was combined with the extract to give a combined extract volume of 8.6L. The aqueous extraction procedure recovered 36% of the available rutin from the leaves. The extract was concentrated under reduced pressure to approximately 1/5 or 1/10 of the original volume. The concentrated extract was stored in the refrigerator (4°C) overnight at which point the flavonoids precipitated out of solution. The precipitated material was collected following centrifugation at 7,000 x g and filtration of the supernatant. The pellet was subsequently freeze-dried. The rutin content of the precipitate was determined by dissolving an aliquot of the dried product in methanol and analyzing by RP-HPLC. From the HPLC results, we have concluded that 60% of the available rutin in the concentrated aqueous extract (reduced to 1/5 and 1/10 of original volume) can be recovered in the precipitate (pellet).

Example 2:

Aqueous Alcohol Extraction, Concentration and Precipitation of Rutin from Buckwheat Leaf Material

Following harvest and drying, buckwheat leaves were prepared for extraction by grinding on a Wiley mill to pass a 2mm screen. One kg of ground buckwheat leaves

(rutin content is 3.74%, dry weight basis) were extracted in 10 L of 50% (v/v) aqueous methanol with continuous stirring at 40°C for 3 hours. The resulting suspension was filtered, and the filtercake was washed with warm (40°C) 50% (v/v) aqueous methanol. The wash filtrate was combined with the extract. The extraction procedure recovered 65% of the available rutin from the leaves. Fig. 2A illustrates the concentration of rutin in the methanol extraction. The extract was concentrated under reduced pressure to approximately 1/5 the original volume. The concentrated extract was stored in the refrigerator (4°C) overnight at which point the flavonoids precipitated out of solution. The precipitated material was collected following centrifugation at 7,000 x g and decantation of the supernatant. The precipitate was subsequently freeze-dried. The rutin content of the precipitate was determined by dissolving an aliquot of the dried product in methanol and analyzing by RP-HPLC. Fig. 2B illustrates the concentration of rutin in the precipitate. The flavonoid-enriched product was found to contain 64% rutin, and 6.88% protein. Rutin recovery of 93 – 100% was demonstrated in the precipitate (pellet) from the concentrated extract.

Example 3:

Purification of Rutin from the Intermediate Flavonoid Enriched Product Isolated from Buckwheat Leaves

The enriched rutin product from Example 2 was dissolved in warm methanol with vigorous stirring on a magnetic stirrer to facilitate complete solubilization of the rutin. Using vacuum filtration, any insoluble material was removed from the solution. The solution was evaporated to dryness at 40°C, under reduced pressure. The residue was then suspended in hot (90°C) water with continuous stirring until most of the precipitate had dissolved. The suspension was allowed to precipitate in the refrigerator overnight. The precipitate was removed by vacuum filtration, and freeze-dried. The purified rutin precipitate was dissolved in methanol, filtered through a 0.45 um nylon syringe filter, and then analyzed by RP-HPLC to determine the purity

of the product. Rutin content can be increased to about 70 % or higher after repeat solubilization/crystallization without using chromatography.

Example 4:

Optimization of Rutin Extraction from Buckwheat Leaf Material

Buckwheat leaves obtained as noted in Example 2 were extracted with the following solvents in a Solid:Solvent ratio of 1:20 for 4 hours at 60°C: Water, 30%(v/v) methanol/ 70% (v/v) water, 50%(v/v) methanol, 70%(v/v) methanol/ 30% (v/v) water, 85%(v/v) methanol/ 15%(v/v) water, and 100% methanol. The resulting extracts were then filtered and analyzed by RP-HPLC. The methanol content in the extraction solvent had a significant effect on the extraction efficiency of rutin from buckwheat leaves (Table 1.)

The optimal extraction conditions for the recovery of rutin from buckwheat leaves were determined from a series of optimization studies. The effects of varying the alcohol content of the extracting solvent, as well as the extraction temperature, extraction time and the solid to solvent ratio were significant. Tables 1-3 summarize some of these results.

Table1: Effect of the concentration of methanol in the extraction solvent on rutin extraction efficiency using 1:20 solid:solvent ratio, and a 4 hour extraction at 60°C.

% methanol in solvent (%, v/v)	Extraction Efficiency of Rutin (%)
0	1.0
30	29.2
50	86.5
70	94.1

85	83.7
100	85.7

[Extraction Efficiency of Rutin (%) = (total rutin in extract / total rutin in the starting material) X 100]

Table 2: Effect of extraction temperature on rutin extraction efficiency using 1:10 solid:solvent ratio, 70%(v/v) Methanol extraction solvent, and a 4 hour extraction.

Extraction Temperature (°C)	Extraction Efficiency of Rutin (%)
24	72.2
30	82.9
40	87.4
50	90.9
60	91.4

[Extraction Efficiency of Rutin (%) = (total rutin in extract / total rutin in the starting material) X100]

Table 3: Effect of extraction time and solid:solvent ratio on rutin extraction efficiency using 70%(v/v) Methanol extraction solvent at 50°C.

Solid:Solvent Ratio	Extraction Time (hrs.)	Extraction Efficiency of Rutin (%)
1:10	2	86.4
1:20	2	91.8
1:30	2	94.5
1:10	3	92.7
1:20	3	92.7
1:30	3	95.4
1:10	4	90.0
1:20	4	99.9
1:30	4	96.3

[Extraction Efficiency of Rutin (%)
= (total rutin in extract / total rutin in the starting material) X 100]

Conversion of Rutin to Isoquercitrin and Quercetin

Fig. 1A illustrates the molecular make-up of rutin. Reaction of the enzyme α-L-rhamnosidase causes a biotransformation from rutin to the isoquercitrin of Fig. 1B by removing the first sugar on the bottom right hand side. To illustrate, the enzyme α-L-rhamnosidase essentially makes a conceptual incision along line A – A' in Fig. 1A.

Reaction of the enzyme β-D-glucosidase causes a biotransformation from the isoquercitrin of Fig. 1B to the quercetin of Fig. 1C by removing the sugar on the

bottom right hand side in Fig. 1B. To illustrate, the enzyme β-D-glucosidase essentially makes a conceptual incision along line B – B' in Fig. 1B.

As shown in Example 5, a composition enriched in isoquercitrin is prepared from the rutin-enriched composition of Example 2 above. The process for doing so comprises providing a solution having rutin suspended therein at conditions suitable for enzyme incubation. These conditions in Example 5 include raising the temperature of the solution to 80°C, and adjusting the pH to 4. An enzyme preparation, namely food-grade naringinase enzyme powder, comprising the enzymes α-L-rhamnosidase and β-D-glucosidase is added to the solution. The conditions of the solution are maintained at those conditions suitable for enzyme incubation during an incubation period with the temperature of the solution at a temperature of 50°C and with continuous stirring.

Changing the conditions of the solution to conditions unsuitable for enzyme incubation terminates the incubation period. In Example 5 this changing included adjusting the pH to 2.5 and then heating to 80°C for ten minutes with continuous stirring.

As seen in Table 4, adjusting the duration of the incubation period controls the proportion of isoquercitrin in the isoquercitrin-enriched composition. The proportion of isoquercitrin increases as the incubation period lengthens with weight ratios of rutin/isoquercitrin/quercetin of 1.71:1:0.06 after 8 hours; 0.33:1:0.07 after 16 hours; and trace:1:0.46 after 24 hours.

Thus after 24 hours, substantially all the rutin has been converted to isoquercitrin and quercetin. After 24 hours however, the composition comprises only approximately twice as much isoquercitrin as quercetin. Prior to this, at 16 hours for instance, the composition comprises approximately fourteen times as much isoquercitrin as quercetin, and three times as much isoquercitrin as rutin.

As the incubation period increases further it can be seen that the isoquercitrin is further converted to quercetin, with the proportion of quercetin to isoquercitrin increasing until at 96 hours, the weight ratios of rutin/isoquercitrin/quercetin in the

composition is trace:1:3.38, and the composition comprises well over three times as much quercetin as isoquercitrin.

It can be readily seen that by adjusting the incubation period the proportions of rutin, isoquercitrin, and quercetin can be adjusted. The incubation times are measured in hours, such that considerable time latitude is available, allowing for conversion on a large scale in commercially significant quantities.

As shown in Example 6, after one day of enzymatic transformation, commercially sourced rutin (purity of 95% by weight) was converted to an isoquercitrin-enriched composition having weight ratios of rutin/isoquercitrin/quercetin of 0.1:1.0:0.2.

As shown in Example 7, commercial rutin was converted from the high rutin composition of Fig. 3A to the high isoquercitrin and quercetin composition of Fig. 3B.

As shown in Example 8, the high isoquercitrin and quercetin composition produced in Example 7 was further purified by Deltaprep C-18 chromatography, and high purity (95% +) isoquercitrin was obtained with a yield of 75% of the isoquercitrin in the starting material.

As shown in example 10, the ß-glucosidase in the naringinase can be inhibited by the addition of D-Δ-gluconolactone, or other food facilitator, without affecting the activity of alpha-rhamnosidase. D-Δ-gluconolactone has been used for years as a food additive, for example as a coagulant in the production of tofu. In the present invention D-Δ-gluconolactone adds flexibility and further assurance that the process will produce high isoquercitrin yield. Selective inhibition of ß-glucosidase, or selective separation of alpha-rhamnosidase from the naringinase for the production of isoquercitrin is within the scope of the claimed invention.

As shown in example 11, a medium-scale process is able to produce a highly enriched isoquercitrin product from buckwheat leaves. Thus, novel products can be produced from low value plant biomass.

Thus a commercially available enzyme mixture, naringinase is used to transform rutin into useful and highly valued flavonoids quercetin and isoquercitrin. The enzymatic transformation herein disclosed is efficient and less expensive than the prior art and does not utilize noxious and potentially harmful solvents. One of the biotransformed products generated from this study has rutin/isoquercitrin/quercetin weight ratios of trace:22.8:7.3. Its composition is similar to the Ginkgo Biloba extract, which typically contains 24.5% flavone glycosides and 6.3% quercetin.

In conjunction with blending products processed under different conditions, products with different chemical profiles can be tailor-made. This technology offers some flexibility for making "designer-nutraceuticals." Furthermore, the converted mixture could be fractionated and purified into high purity compounds using chromatography or other techniques.

Although chromatographic methods for the separation of flavonoids are described in the literature, they are principally designed for analytical purposes. The purification of enzyme-converted flavonoids (rutin, isoquercitrin and quercetin mixtures) using Stack Pack Columns, which would handle 5-50 liters of extract, is not previously known.

As shown in Example 9, the biotransformation technology disclosed by the invention also can convert rutin to isoquercitrin and quercetin in St. John's Wort. Various other biomasses such as ginkgo biloba, alfalfa, mulberry leaves etc. as well as other rutin-enriched agricultural biomass such as rose hips, apple peels, pear peels, onion skins, and asparagus tips also contain rutin and could be used to produce the isoquercitrin-enriched composition.

Quercetin and isoquercitrin demand higher prices because of rareness and bioavailability/bioefficacy. The increased bioavailability of quercetin and isoquercitrin in cardiovascular disease and cancer prevention suggests a promising role of converted flavonoid product in the nutraceutical and pharmaceutical markets.

Example 5:

Conversion of Rutin to Isoquercitrin and Quercetin using Enzymatic Hydrolysis

By manipulating the biotransformation conditions, we were able to convert the flavonoid-enriched intermediates to products containing different profiles of rutin/isoquercitrin/quercetin.

The freeze-dried rutin product (approximately 60% Rutin) produced in Example 2 was used for the enzymatic conversion experiments. A quantity of 5 grams of dry rutin product was dispersed in 500 ml of water (solid:liquid ratio = 1:100). The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 50°C, followed by addition of food-grade naringinase enzyme powder (Amano Pharmaceutical Co., Ltd; Japan).

The naringinase preparation contains 150 units of beta-glucosidase or naringinase activity as described in the specifications from the supplier. A dosage of 66 mg of Amano naringinase was used per g of rutin in this trial. The enzymatic incubation was maintained at 50°C with continuous stirring, for the appropriate length of time. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and then heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room temperature, and the pH adjusted to 7. The enzyme-converted product was then dried by spray drying, freeze drying or other appropriate means.

Table 4 summarizes the experimental conditions required to prepare products containing various rutin/isoquercitrin/quercetin profiles. The starting material described here was previously freeze-dried for convenience reason. The precipitate (pellet) recovered prior to the drying step in the Example 2 is also suitable as a starting material for the Example 5. The enzymatic conversion can be applied at different stages, i.e., prior to the extraction of flavonoids, after aqueous extraction, after pre-concentration, or after precipitation. Flavonoid profiles

(rutin/isoquercitrin/quercetin) remains unchanged in the control (without enzyme) following the identical procedures to the normal enzymatic treatment. This indicates that the transformation was caused by the action of naringinase.

Table 4: Enzymatic conversion of flavonoids with naringinase.

Trial #	Enzyme Dosage (mg enzyme/g Rutin Ppt.)	Incubation Time (hrs.)	Incubation Temperature (°C)	Weight Ratio of Rutin:Isoquercitrin:Quercetin
1	66	8	50	1.71:1:0.06
2	66	16	50	0.33:1:0.07
3	66	24	50	trace:1:0.46
4	66	48	50	trace:1:0.72
5	66	72	50	trace:1:1.65
6	66	96	50	trace:1:3.38

Example 6:

Conversion of High Purity Commercial Rutin to Isoquercitrin

Using commercial rutin (95% Purity), purchased from Sigma Chemical Company, an enzyme incubation similar to that described in Example 5, was performed in order to convert the rutin to isoquercitrin. A quantity of 10.90g of rutin was dispersed in 1000 ml water. The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 55°C, followed by addition of 2.42g of naringinase enzyme powder. The enzymatic incubation was maintained at 55°C with continuous stirring, for 24 hours. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room

temperature, and the pH adjusted to 7. A 1.0 ml aliquot of the extract was removed for RP-HPLC analysis of the composition of the final product. The remaining extract was freeze-dried. The HPLC results indicated that the pure rutin standard had been converted to a product containing a rutin/isoquercitrin/quercetin profile of 0.12:1:0.21 (weight ratio).

Example 7:

Scale-up Conversion

Commercial rutin purchased from Street Chemicals was used for the enzymatic conversion similar to that described in Example 6. Concentrations of rutin and isoquercitrin in the commercial rutin are shown in Fig. 3A. A quantity of 109g of rutin was dispersed in 4000 ml water. The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 55°C, followed by addition of 24.2g of naringinase enzyme powder. The enzymatic incubation was maintained at 55°C with continuous stirring, for 24 hours. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room temperature, and the pH adjusted to 7. The solution was stored in the refrigerator (4°C) overnight. The solids recovered from centrifugation were freeze-dried. A quantity of 61.8 g of dry matter was obtained. The chromatogram of this product is shown in the Fig 3B.

Example 8: Preparative Scale Isolation of Isoquercitrin and Quercetin

The solids (50gm) obtained from the method of example 7 were dissolved in 70% methanol and filtered. The resulting extract was subjected to preparative scale chromatography on a Waters reversed phase Bondapak C-18, 40x310mm (15-20 125Å) column eluted with a Methanol:1% acetic acid gradient at a flow rate of 50 ml/min using a Waters Delta-Prep 4000 system equipped with a 486 variable wavelength UV-Vis detector controlled by Millennium V 2.15 software. The compounds of interest were detected at 280 nm. The purity of the fractions collected were evaluated using the analytical HPLC procedure described in example A .The yield of isoquercitrin was 75% of the starting material with a 95% purity (Figure 3C). A small quantity of pure quercetin was also recovered from one of the Preparative HPLC fractions. The purity of the preparative HPLC fractions could be further improved by re-crystallization from hot methanol.

Example 9:
Conversion of Rutin from St. John's Wort Extract

The contents of several St. John's Wort capsules were combined, dispersed in water, and subjected to an enzyme incubation similar to that described in Example 5. St. John's Wort is known to contain rutin. The objective of this experiment was to convert the rutin present in the St. John's Wort extract to isoquercitrin. A quantity of 5.52g of St. John's Wort extract was dispersed in 500 ml water. The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 55°C, followed by addition of 0.60g of naringinase enzyme powder. The enzymatic incubation was maintained at 55°C with continuous stirring, for 24 hours. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room temperature, and the pH adjusted to 7. The extract was then freeze-dried. The dried product was dissolved in methanol, filtered and analyzed by RP-HPLC to determine the extent of the conversion of rutin

to isoquercitrin. The HPLC results of the initial St. John's Wort extract indicated a rutin/isoquercitrin/quercetin profile of 0.47:1:0.21 (weight ratio). The enzyme converted product was found to contain a rutin/isoquercitrin/quercetin profile of trace:1:0.18 (weight ratio) which indicated that all rutin present in the initial extract had been converted to isoquercitrin and quercetin.

Example 10: Large-scale conversion of rutin to isoquercitrin using naringinase and D-delta- gluconolactone

Pharmaceutical grade rutin (38.15 g) from ICN was dispersed in 3.5 L of deionized water. Naringinase (8.47 g in 100 ml water) and D-Δ-gluconolactone (6.23 g in 100 ml water) solution were prepared. D- Δ-gluconolactone solution as added to the rutin:water mixture. The pH of the mixture was 4.0. The mixture was then heated to 80°C and incubated 2 hr. The temperature was then reduced to 55°C and the naringinase solution added. The mixture was incubated for 24 hr at 55°C with stirring. To stop the reaction, the pH was decreased to 2.5 and the mixture heated to 80°C for 10 min. The mixture was allowed to cool to room temperature and then the pH was adjusted to 7.0. The mixture was then refrigerated overnight to induce formation of a precipitate and the precipitate was allowed to settle. The precipitate was collected by centrifugation and then freeze-dried (the PPT1 fraction). The supernatant fluid was concentrated and then re-centrifuged. The resultant pellet was also freeze-dried (the PPT2 fraction). Three batches were prepared in this manner. The rutin, isoquercitrin and quercetin in different fractions from each batch were analyzed by HPLC. The data are presented in Table 5.

The data in table 5 demonstrates that rutin and quercetin appear as minor components, whereas isoquercitrin is the principal product observed after enzymatic conversion. For example, a total of 77.72 g of isoquercitrin and 0.53 of quercetin were produced from the three batches that were processed by the method of example 10. The majority (61.8 g) of isoquercitrin appeared in the PPT 1 fraction. The conversion process was very efficient, as only 0.2009 g of rutin was left unconverted by the

process. The data also shown that the inclusion of the inhibitor D-delta-gluconolactone to the reaction mixture selectively results in the production of primarily one type of flavinoid, namely isoquercitrin.

Isoquercitrin concentration in the PPT1 fraction varied from averaged 85.2% (the range was 81.1 – 88.8%). This example therefore, also shows that high purity (> 80%) bioavailable flavinoids can be produced using simple biochemical purification techniques, and without the need for chromatographic methods. The supernatant (SUP) fractions were also valuable as they contained 14.42 g of isoquercitrin per 101.66 g of dry matter.

Table 5: Enzymatic conversion of flavinoids with naringinase and D-delta-gluconolactone as a selective inhibitor.

Batch #	Total Freeze-dried weight (g)	Total Rutin (g)	Total Isoquercitrin (g)	Total Quercetin (g)
1				
PPT1	23.23	0.0495	18.8495	0.5299
PPT2	0.54	0.0054	0.2105	--
SUP	36.41	0.0844	6.9944	--
2				
PPT1	26.30	0.0604	23.3768	--
PPT2	0.64	--	0.2828	--
SUP	30.64	--	2.3697	--
3				
PPT1	22.92	--	19.6154	--
PPT2	1.34	0.0012	0.9727	0.0008
SUP	34.61	--	5.0559	--

Example 11: Extraction, Conversion and Purification of Rutin from Buckwheat Leaf Materials

One kg of ManCan leaf material was extracted in 10 L of 70% methanol for 3 hr at 50°C. After 3 hr, the mixture was filtered and the plant material washed with

approximately 4 L of hot 70% methanol. The filtrates were combined and the volume reduced using a rotary evaporator until the volume was 1/5 of the original volume. The concentrated extract was refrigerated and allowed to precipitate overnight. The mixture was then stirred, and then centrifuged to collect the rutin.

Based on previous analysis, rutin content was estimated to be 33.66 g from 1 kg of starting leaf material. The amounts of enzyme and inhibitor used were based on these estimates, and were similar to previous conversions (7.36 g naringinase; 6.23 g D-Δ-gluconolactone; 3.5 L water).

The rutin precipitate was added to 3.5 L of water, and D-Δ-gluconolactone solution added. The pH of the mixture was 4.0. The mixture was then heated to 80°C and incubated for 2 hr. The mixture was then cooled to 55°C and the naringinase solution added. The mixture was then incubated at 55°C for 24 hrs. The reaction was stopped by reducing the pH to 2.5, and then incubating at 80°C for 10 min. The mixture was cooled to room temperature and the pH adjusted to 7.0. The mixture was then placed at 4°C overnight to allow a precipitate to form. The precipitate was collected by centrifugation as in Example 10.

The precipitate pellet was dissolved in methanol at 55°C with stirring. The solution was filtered to remove insoluble material. The filtrate was then concentrated as much as possible without allowing the mixture to bubble in the concentration vessel. At this point, 1.5 L of hot water was added to the mixture, and the material re-precipitated by incubation at 4°C for 2 days, and the precipitate collected by centrifugation. The re-precipitated material was then washed with hot water and precipitated for a third time. This final precipitate was freeze-dried to form a final product.

Note Respecting Methods and Examples

The buckwheat flavonoid content was determined by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters Symmetry C-18 column

(3.0.x.150mm, 5 micrometer) eluted with a linear gradient of aqueous 0.05% v/v trifluoracetic acid (TFA): acetonitrile (T=0 min., % acetonitrile=10; T=20min., % acetonitrile=40; T=30min., % acetonitrile=10) at a flow rate of 0.4 mL/min, with photodiode array (PDA) detector at 350 nm. Quantification of flavonoids was by external standard curves using rutin, isoquercitrin and quercetin standards purchased commercially.

Rutin in the biomass was extracted out by solvent and determined by the HPLC method as described by Minami et al (1998). One gram of biomass (passed through a 100 mesh screen) was extracted with 40 ml of methanol at 70°C for 60 min. in a Soxhelt extraction apparatus. The supernatant after centrifugation was used for the determination.

As summarized in Fig. 4, the present invention provides for the extraction, concentration, and precipitation of rutin enriched compositions from rutin containing plant biomass, and enzymatic conversion of rutin to isoquercitrin/quercetin enriched compositions using method A, or alternatively, the production of a isoquercitrin-enriched product. Both the products of method A or B of the invention are useful as additives for the health food, nutraceutical, pharmaceutical, cosmetic and other markets.

The foregoing is considered as illustrative only of the principles of the invention. Further, since numerous changes and modifications will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described, and accordingly, all such suitable changes or modifications in structure or operation which may be resorted to are intended to fall within the scope of the claimed invention.

Claims

We claim:

1. An isoquercitrin-enriched composition prepared by a process comprising:

providing a solution having rutin suspended therein at conditions suitable for enzyme incubation;

adding an enzyme preparation comprising naringinase to the solution;

maintaining the conditions of the solution suitable for enzyme incubation during an incubation period;

terminating the incubation period by changing the conditions of the solution to conditions unsuitable for said enzyme incubation;

wherein the proportion of isoquercitrin in the composition is controlled by adjusting the duration of the incubation period.

2. The isoquercitrin-enriched composition of Claim 1 wherein the composition is further enriched with quercetin as a result of the enzyme incubation.

3. The isoquercitrin-enriched composition of Claim 2 wherein the relative proportion of quercetin and isoquercitrin is controlled by adjusting the duration of the incubation period.

4. The isoquercitrin-enriched composition of Claim 2 wherein the duration of the incubation period is dependent on the activity of the enzyme preparation.

5. The isoquercitrin-enriched composition of Claim 2 wherein the duration of the incubation period is in the range of 1 – 48 hr.

6. The isoquercitrin-enriched composition of Claim 1 wherein the conditions of the solution during enzyme incubation include temperature and pH level.

7. The isoquercitrin-enriched composition of Claim 6 wherein the temperature is in the range of 50 – 55°C.

8. The isoquercitrin-enriched composition of Claim 6 wherein the pH is in the range of 4 – 8.

9. The isoquercitrin-enriched composition of Claim 1 wherein the conditions of solution are an acidic pH and a temperature of substantially 80°C.

10. The isoquercitrin-enriched composition of Claim 1 wherein the ratio of rutin to isoquercitrin is less than 20:1 by weight.

11. The isoquercitrin-enriched composition of Claim 2 wherein the ratio of quercetin to isoquercitrin is greater than 0.003:1 by weight.

12. The isoquercitrin-enriched composition of Claim 1 wherein said process further comprises purification of said solution following termination of said incubation period.

13. The isoquercitrin-enriched composition of Claim 12 wherein the purification of said solution following termination of said incubation period is conducted using conventional biochemical purification.

14. A purified isoquercitrin composition comprising at least 90% isoquercitrin by weight prepared by subjecting the isoquercitrin-enriched composition of Claim 1 to conventional biochemical purification methods.

15. The isoquercitrin-enriched composition of Claim 1 wherein the rutin is obtained in enriched or purified form from a commercial source.

16. The isoquercitrin-enriched composition of Claim 1 wherein the rutin is obtained by the method of Claim 53.

17. An isoquercitrin-enriched composition containing isoquercitrin produced in accordance with the process of Claim 1, said composition having bioactive properties comprising angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory, tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

18. The isoquercitrin-enriched composition of Claim 17, wherein the bioactive properties of said composition are used in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

19. The isoquercitrin-enriched composition of Claim 17, wherein said composition is used in functional foods.

20. The isoquercitrin-enriched composition of Claim 17, wherein said composition is used in natural health products.

21. The isoquercitrin-enriched composition of Claim 17, wherein said composition is used in nutraceutical products.

22. The isoquercitrin-enriched composition of Claim 17, wherein said composition is used in pharmaceutical products.

23. The isoquercitrin-enriched composition of Claim 17, wherein said composition is used in cosmetic products.

24. An isoquercitrin-enriched composition prepared by a process comprising:

providing a solution having rutin suspended therein at conditions suitable for enzyme incubation;

adding an enzyme preparation comprising naringinase to the solution;

maintaining the conditions of the solution suitable for enzyme incubation during an incubation period;

terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme incubation;

wherein the proportion of isoquercitrin in the composition is controlled by adjusting the duration of the incubation period.

24. The isoquercitrin-enriched composition of Claim 24, wherein the yield of isoquercitrin is controlled by adjusting the duration of the incubation period.

25. The method of Claim 24, wherein the duration of the incubation period is in the range of 1 – 48 hr.

26. The isoquercitrin-enriched composition of Claim 24 wherein the relative proportion of rutin, quercetin and isoquercitrin is controlled by the addition of a ß-D-glucosidase inhibitor to the solution.

27. The isoquercitrin-enriched composition of Claim 26 wherein said ß-D-glucosidase inhibitor is added to the solution before the addition of said enzyme preparation to the solution

28. The isoquercitrin-enriched composition of Claim 26 wherein the ß-D-glucosidase inhibitor has the properties of D-Δ-gluconolactone.

29. The isoquercitrin-enriched composition of Claim 28 wherein the ß-D-glucosidase inhibitor is D-Δ-gluconolactone.

30. The isoquercitrin-enriched composition of Claim 24 wherein the enzyme preparation comprises α-L-rhamnosidase.

31. The isoquercitrin-enriched composition of Claim 24 wherein the conditions of the solution during enzyme incubation include temperature and pH level.

32. The isoquercitrin-enriched composition of Claim 31 wherein the temperature is in the range of 50 – 55°C.

33. The isoquercitrin-enriched composition of Claim 31 wherein the pH is in the range of 4 – 8.

34. The isoquercitrin-enriched composition of Claim 24 wherein the conditions of the solution during enzyme incubation includes the addition of a ß-D-glucosidase inhibitor.

35. The isoquercitrin-enriched composition of Claim 34, wherein the ß-D-glucosidase inhibitor has the properties of D-Δ-gluconolactone.

36. The isoquercitrin-enriched composition of Claim 25 wherein the ß-D-glucosidase inhibitor is D-Δ-gluconolactone.

37. The method of Claim 35 wherein the concentration of D-Δ-gluconolactone is greater than 1 mM.

38. The isoquercitrin-enriched composition of Claim 24 further comprising terminating the incubation period by denaturing the enzyme α-L-rhamnosidase.

39. The isoquercitrin-enriched composition of Claim 24 wherein the rutin is obtained in enriched or purified form from a commercial source.

40. The isoquercitrin-enriched composition of Claim 24 wherein the rutin is obtained by the method of Claim 53.

41. The isoquercitrin-enriched composition of Claim 24 wherein the ratio of rutin to isoquercitrin is less than 20:1 by weight.

42. The isoquercitrin-enriched composition of Claim 24 wherein the ratio of quercetin to isoquercitrin is greater than 0.003:1 by weight.

43. The isoquercitrin-enriched composition of Claim 24 wherein said process further comprises purification of said solution following termination of said incubation period.

44. The isoquercitrin-enriched composition of Claim 43 wherein the purification of said solution following termination of said incubation period is conducted using conventional biochemical purification.

45. A purified isoquercitrin composition comprising at least 90% isoquercitrin by weight prepared by subjecting the isoquercitrin-enriched composition of Claim 24 to conventional biochemical purification.

46. An isoquercitrin-enriched composition containing isoquercitrin produced in accordance with the process of Claim 1, said composition having bioactive properties comprising angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory, tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

47. The isoquercitrin-enriched composition of Claim 46, wherein the bioactive properties of said composition are used in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

48. The isoquercitrin-enriched composition of Claim 46, wherein said composition is used in functional foods.

49. The isoquercitrin-enriched composition of Claim 46, wherein said composition is used in natural health products.

50. The isoquercitrin-enriched composition of Claim 46, wherein said composition is used in nutraceutical products.

51. The isoquercitrin-enriched composition of Claim 46, wherein said composition is used in pharmaceutical products.

52. The isoquercitrin-enriched composition of Claim 46, wherein said composition is used in cosmetic products.

53. A process for preparing a rutin enriched composition from biomass containing rutin, the process comprising:

performing a flavonoid extraction process on the biomass using an aqueous solution comprising water or alcohol;

filtering the solution to produce an extract solution;

allowing the extract solution to stand such that a precipitate forms;

collecting and drying the precipitate to form the rutin enriched composition.

54. The process of Claim 53 wherein the flavonoid extraction process comprises fragmenting the biomass and agitating it in the aqueous solution.

55. The process of Claim 53 further comprising concentrating the extract solution to form a concentrated extract solution having less than one fifth of its original volume prior to allowing the extract solution to stand.

56. The process of Claim 55 wherein the concentrated extract solution is allowed to stand at a temperature less than 10°C.

57. The process of Claim 53 wherein the aqueous solution comprises water and is maintained at a temperature above 30°C.

58. The process of Claim 53 wherein the aqueous solution comprises alcohol.

59. The process of Claim 58 wherein the aqueous solution has a concentration of greater than 20% alcohol by volume, and the balance of the solution is water.

60. The process of Claim 59 wherein the aqueous solution has a concentration of between 50% and 100% alcohol by volume, and the balance of the solution is water.

61. The process of Claim 59 wherein the temperature of the aqueous solution is maintained at between 30°C and 99°C during the extraction process.

62. The process of Claim 53 wherein the plant biomass comprises biomass from a member of the genus of *Fargopyrum*.

63. The process of Claim 53 wherein the biomass comprises at least one of: leaves of St. John's Wort; ginkgo; biloba; alfalfa; mulberry; algae; apple peels; pear peels; onion skins; asparagus tips; and rose hip pericarps.

64. A flavonoid-enriched composition containing rutin produced in accordance with the process of Claim 53, said composition having bioactive properties comprising angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory, tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

65. The flavonoid-enriched composition of Claim 64, wherein the bioactive properties of said composition are used in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

66. The flavonoid-enriched composition of Claim 64, wherein said composition is used in functional foods.

67. The flavonoid-enriched composition of Claim 64, wherein said composition is used in natural health products.

68. The flavonoid-enriched composition of Claim 64, wherein said composition is used in nutraceutical products.

69. The flavonoid-enriched composition of Claim 64, wherein said composition is used in pharmaceutical products.

70. The flavonoid-enriched composition of Claim 64, wherein said composition is used in cosmetic products.

71. A method of producing an isoquercitrin-enriched composition, said method comprising:

providing a solution having rutin suspended therein at conditions suitable for enzyme incubation;

adding an enzyme preparation comprising naringinase to the solution;

maintaining the conditions of the solution suitable for enzyme incubation during an incubation period;

terminating the incubation period by changing the conditions of the solution to conditions unsuitable for said enzyme incubation, said solution at this point being an isoquercitrin-enriched composition;

wherein the proportion of isoquercitrin in the composition is controlled by adjusting the duration of the incubation period.

72. The method of Claim 71 wherein said composition also contains quercetin as a result of said enzyme incubation.

73. The method of Claim 72 wherein the relative proportion of quercetin and isoquercitrin is controlled by adjusting the duration of the incubation period.

74. The method of Claim 71 wherein the duration of the incubation period is dependent on the activity of the enzyme preparation.

75. The method of Claim 71 wherein the duration of the incubation period is in the range of 1 – 48 hr.

76. The method of Claim 71 wherein the conditions of the solution during enzyme incubation include temperature and pH level.

77. The method of Claim 76 wherein the temperature is in the range of 50 – 55°C.

78. The method of Claim 76 wherein the pH is in the range of 4 – 8.

79. The method of Claim 71wherein the conditions of solution are an acidic pH and a temperature of substantially 80°C.

80. The method of Claim 71 wherein the ratio of rutin to isoquercitrin is less than 20:1 by weight.

81. The method of Claim 80 wherein the ratio of quercetin to isoquercitrin is greater than 0.003:1 by weight.

82. The method of Claim 71 further comprising purification of said solution following termination of said incubation period.

83. The method of Claim 82 wherein the purification of said solution following termination of said incubation period is conducted using conventional biochemical purification.

84. The product, purified isoquercitrin, manufactured by the method of Claim 83.

85. An isoquercitrin-enriched composition containing isoquercitrin produced in accordance with the method of Claim 71, said composition having bioactive properties comprising angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory, tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

86. The isoquercitrin-enriched composition of Claim 85, wherein the bioactive properties of said composition are used in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

87. The isoquercitrin-enriched composition of Claim 85, wherein said composition is used in functional foods.

88. The isoquercitrin-enriched composition of Claim 85, wherein said composition is used in natural health products.

89. The isoquercitrin-enriched composition of Claim 85, wherein said composition is used in nutraceutical products.

90. The isoquercitrin-enriched composition of Claim 85, wherein said composition is used in pharmaceutical products.

91. The isoquercitrin-enriched composition of Claim 85, wherein said composition is used in cosmetic products.

92. A method of producing an isoquercitrin-enriched composition, said method comprising:

providing a solution having rutin suspended therein at conditions suitable for enzyme incubation;

adding an enzyme preparation comprising naringinase to the solution;

maintaining the conditions of the solution suitable for enzyme incubation during an incubation period;

terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme incubation;

wherein the proportion of isoquercitrin in the composition is controlled by adjusting the duration of the incubation period.

93. The method of Claim 92, wherein the yield of isoquercitrin is controlled by adjusting the duration of the incubation period.

94. The method of Claim 92, wherein the duration of the incubation period is in the range of 1 – 48 hr.

95. The method of Claim 92 further comprising the addition of a ß-D-glucosidase inhibitor to the solution to control the relative proportion of rutin, quercetin and isoquercitrin in the solution.

96. The method of Claim 95 wherein said ß-D-glucosidase inhibitor is added to the solution before the addition of said enzyme preparation to the solution

97. The method of Claim 95 wherein the ß-D-glucosidase inhibitor has the properties of D-Δ-gluconolactone.

98. The method of Claim 97 wherein the ß-D-glucosidase inhibitor is D-Δ-gluconolactone.

99. The method of Claim 92 wherein the enzyme preparation comprises α-L-rhamnosidase.

100. The method of Claim 92 wherein the conditions of the solution during enzyme incubation include temperature and pH level.

101. The method of Claim 100 wherein the temperature is in the range of 50 – 55°C.

102. The method of Claim 100 wherein the pH is in the range of 4 – 8.

103. The method of Claim 92 wherein the conditions of the solution during enzyme incubation includes the addition of a ß-D-glucosidase inhibitor.

104. The method of Claim 103, wherein the ß-D-glucosidase inhibitor has the properties of D-Δ-gluconolactone.

105. The method of Claim 104 wherein the ß-D-glucosidase inhibitor is D-Δ-gluconolactone.

106. The method of Claim 105 wherein the concentration of D-Δ-gluconolactone is greater than 1 mM.

107. The method of Claim 92 further comprising terminating the incubation period by denaturing the enzyme α-L-rhamnosidase.

108. The method of Claim 92 wherein the ratio of rutin to isoquercitrin is less than 20:1 by weight.

109. The method of Claim 92 wherein the ratio of quercetin to isoquercitrin is greater than 0.003:1 by weight.

110. The method of Claim 92 further comprising purification of said solution following termination of said incubation period.

111. The method of Claim 110 wherein the purification of said solution following termination of said incubation period is conducted using conventional biochemical purification.

112. The product, purified isoquercitrin, produced in accordance with the method of Claim 111.

113. An isoquercitrin-enriched composition containing isoquercitrin produced in accordance with the process of Claim 92, said composition having bioactive properties comprising angiotensin-converting enzyme inhibitory, anti-inflammatory, anti-tumor, anti-viral, anti-oxidative, free radical scavenging, cancer preventative, cardioprotective, proteinase-inhibitory, protein kinase C inhibitory, tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

114. The isoquercitrin-enriched composition of Claim 113, wherein the bioactive properties of said composition are used in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

115. The isoquercitrin-enriched composition of Claim 113, wherein said composition is used in functional foods.

116. The isoquercitrin-enriched composition of Claim 113, wherein said composition is used in natural health products.

117. The isoquercitrin-enriched composition of Claim 113, wherein said composition is used in nutraceutical products.

118. The isoquercitrin-enriched composition of Claim 113, wherein said composition is used in pharmaceutical products.

119. The isoquercitrin-enriched composition of Claim 113, wherein said composition is used in cosmetic products.

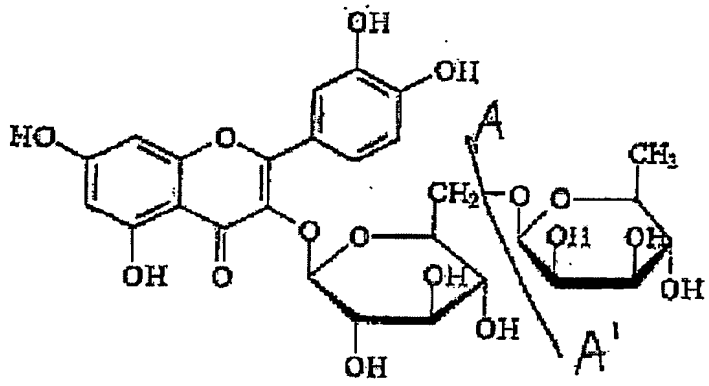


Fig. 1A

Rutin (Quercetin-3-rutinoside)

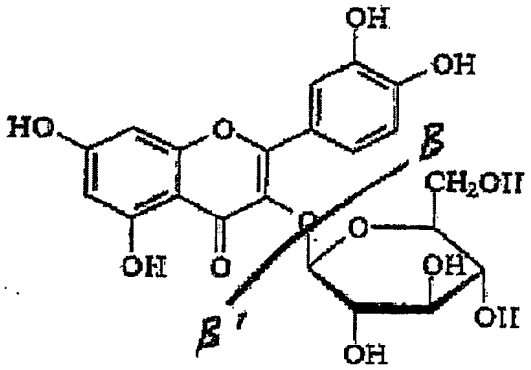


Fig. 1B

Isoquercitrin (Quercetin-3-glucoside)

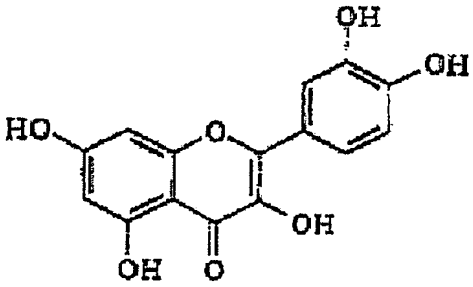


Fig. 1C

Quercetin

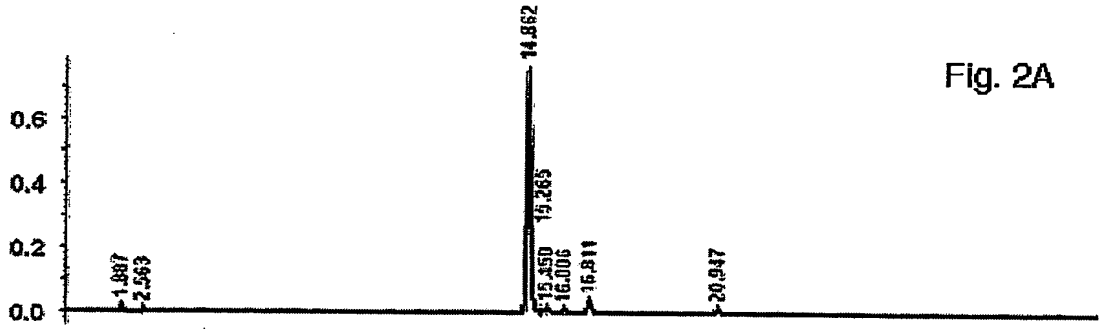


Fig. 2A

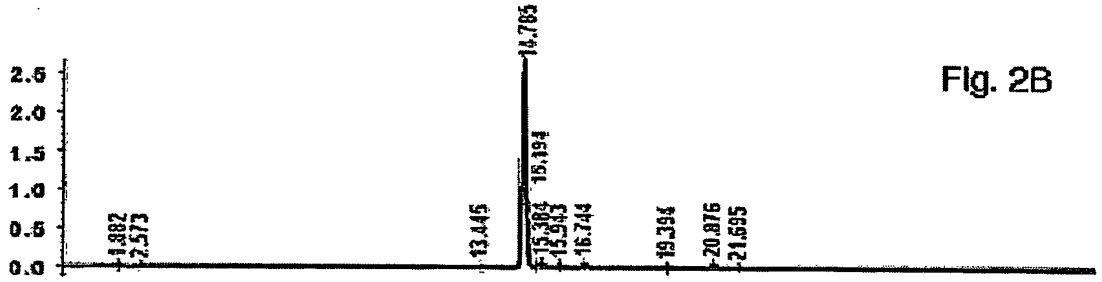


Fig. 2B

Fig. 2C

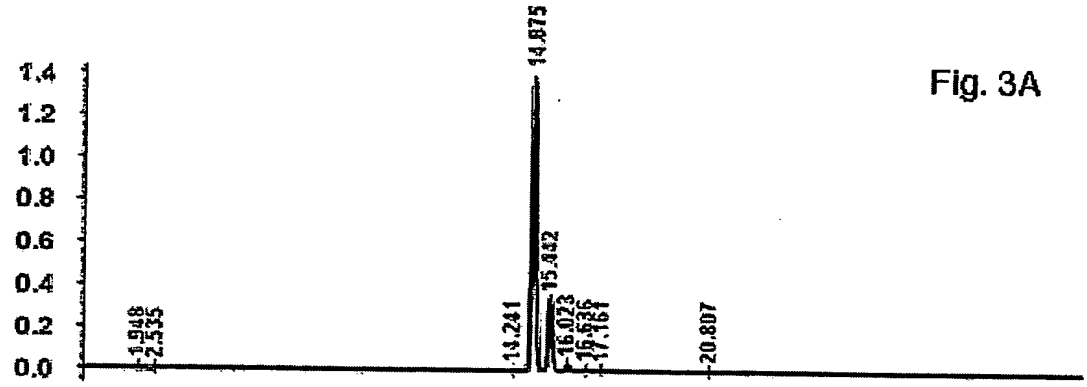


Fig. 3A

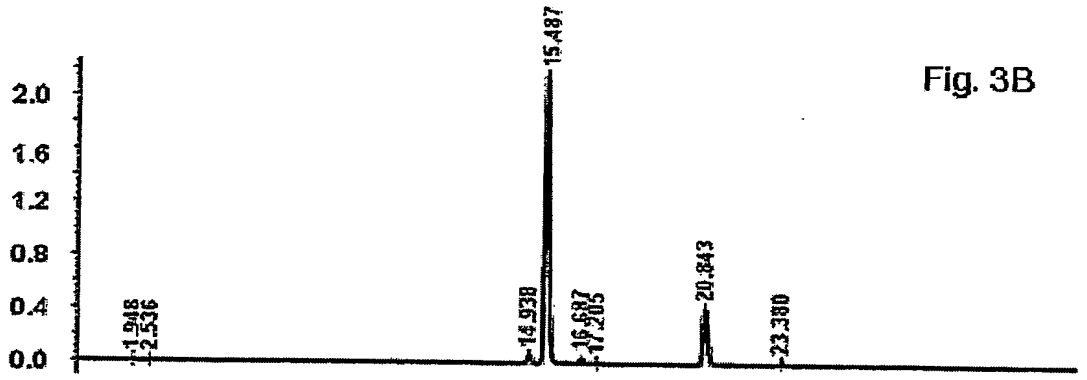


Fig. 3B

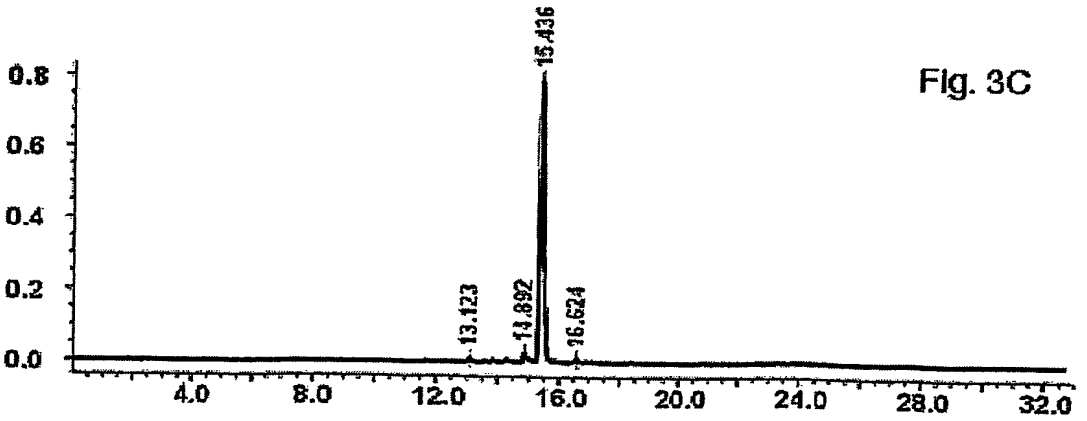
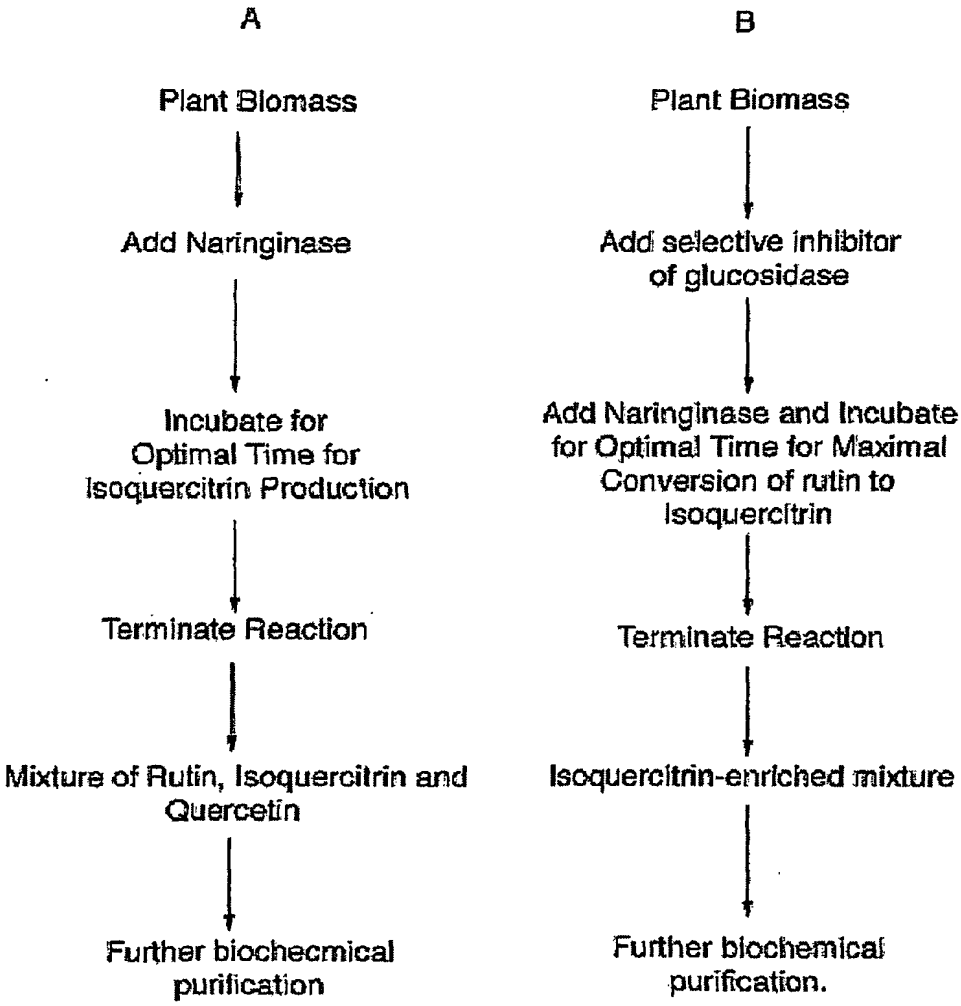


Fig. 3C

Fig. 4


A
Plant Biomass
Add Naringinase
Incubate for Optimal Time for Isoquercitrin Production
Terminate Reaction
Mixture of Rutin, Isoquercitrin and Quercetin
Further biochecmical purification
B
Plant Biomass
Add selective inhibitor of glucosidase
Add Naringinase and Incubate for Optimal Time for Maximal Conversion of rutin to Isoquercitrin
Terminate Reaction
Isoquercitrin-enriched mixture
Further biochemical purification.

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: EXTRACTION, PURIFICATION AND CONVERSION OF FLAVONOIDS FROM PLANT BIOMASS

(57) Abstract: A process for preparing a rutin-enriched composition from plant biomass comprises extraction with an aqueous solution, and precipitation. An enzyme preparation, such as naringinase, is used for the transformation of rutin to higher value compositions containing increased proportions of isoquercitrin and quercetin.

WO 2004/027074 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted]3/01453

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/60 C12P17/06 C07H15/26 C07D311/30 A23L1/00
A61K7/00 A61K31/352 A61K31/7048 C07H17/07

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07H C07D A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 08, 29 August 1997 (1997-08-29) & JP 9 094077 A (SANEI GEN F F I INC), 8 April 1997 (1997-04-08) abstract	1-52, 71-111
X	WO 00/26400 A (MERCK PATENT GMBH) 11 May 2000 (2000-05-11) see especially p. 13-15 the whole document	1-52, 71-111
X	WO 01/59143 A (MERCK PATENT G.M.B.H., GERMANY MERCK PATENT G.M.B.H., GERMANY) 16 August 2001 (2001-08-16) see especially p. 3, 8 and 30 and claims 1-4 the whole document	1-52, 71-111

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2004	04.08.04

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Douschan, K

INTERNATIONAL SEARCH REPORT

International Application No
PCT/C[redacted]3/01453

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/51482 A (BIOREX HEALTH LIMITED, AUSTRALIA BIOREX HEALTH LIMITED, AUSTRALIA) 19 July 2001 (2001-07-19) see especially p. 6 and claim 27 the whole document	71-83
X	US 5 122 381 A (HORIKAWA HIROSHI ET AL) 16 June 1992 (1992-06-16) cited in the application the whole document	1-52, 84-91, 112-119

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/CA 03/01453

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-52, 71-119

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-52, 71-119

Process for preparing an isoquercitrin-enriched composition by incubating rutin suspended in a solution with naringinase (possibly with additional alpha-L-rhamnosidase) and possibly by addition of a beta-D-glucosidase inhibitor, and compositions obtainable from the said process.

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2. claims: 53-70

Process for preparing a rutin-enriched composition from biomass containing rutin by an extraction, filtration and precipitation process, and compositions obtainable by the said process.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/C[redacted]/01453

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